III. Specification Amendments

(On page 1, line 2, please insert as a new ¶)

Related Applications

The present application is a divisional of USSN 09/387,693, filed August 31, 1999, which is a divisional of USSN 09/057,570, filed April 9, 1998, now US Pat. No. 6,013,266.

(On page 17, line 7, please replace all paragraphs until the end of Example 8 on page 21)

On the basis of the preliminary sequence data obtained from the thesis of T.J. Anderson (University of Guelph, 1995), two primers, designated APXIVA-1F. (5'-TGGCACTGACGGTGATGA-3') (SEQ ID NO: 8) and APXIVA-1R (5'-GGCCATCGACTCAACCAT-3') (SEQ ID NO: 14), were synthesised. These primers were used in a PCR amplification, with chromosomal DNA from *A. pleuropneumoniae* serotype 3 strain HV114 and serotype 1 reference strain 4074 as a template. With both strains a fragment of 442 bp was amplified. The fragment derived from the serotype 3 chromosomal DNA was labelled with Digoxigenin-11-dUTP (Boehringer Mannheim) according to the protocol of the manufacturer (this fragment was designated probe APXIVA, see fig. 4). The labelled probe was subsequently used to hybridize a Southern blot containing *Cla*I digested chromosomal DNA from strain 4074. The probe hybridised with a fragment of approximately 8.0 kb. The *apxIV* gene from serotype 1 strain 4074 was isolated by ligating *Cla*I digested chromosomal DNA into *Cla*I digested pBluescript II SK⁻ (Stratagene USA). *E. coli* strain XL1-blue was transformed with the ligated DNA and transformants were selected on an LB plate with 100 mg/ml of ampicillin. Clones harbouring the *apxIV* were selected by colony hybridisation of a nitrocellulose replica of the plate with the APXIVA probe. Thus, a plasmid designated pROK7 was isolated and shown to harbour a *Cla*I insert of approximately 8 kb. The first 6736 bp of the *Cla*I insert were sequenced

(SEQID 1) and an open reading frame of 4971 nucleotides was identified encoding a protein of 1657 amino acid residues (SEQID 2) with a predicted size of approximately 186 kD. The gene was designated *apxIV_var1* (see fig. 3).

Example 2:

Cloning and analysis of the *apxIV* gene of A. pleuropneumoniae serotype 3.

The labelled probe APXIVA (mentioned in example 1) was used to hybridize a Southern blot containing *Cla*I digested chromosomal DNA from strain HV114. The probe hybridised with a fragment of approximately 7.0 kb. The isolated chromosomal DNA from HV114 was digested with *Cla*I, and ligated with *Cla*I digested pBluescript II SK⁻ (Stratagene USA). *E. coli* strain XL1-blue was transformed with the ligated DNA and transformants were selected on an LB plate with 100 mg/ml of ampicillin. Clones harbouring the *apxIV* were selected by colony hybridisation of a nitrocellulose replica of the plate with the APXIVA probe. Thus, a plasmid designated pROK5 was isolated and shown to harbour a *Cla*I insert of approximately 7 kb. The insert was analysed by sequence analysis (SEQID 3). An open reading frame of 4146 bp was identified encoding a protein of 1382 amino acid residues (SEQID 4), with a predicted size of approximately 154 kD. The gene was designated *apxIV_var3* (see fig. 3).

Example 3:

EXPRESSION OF ApxIV_var3-polyhistidine fusion proteins in *E. coli*

From plasmid pROK5, a deletion clone was made which contains the 3' end of the *apxIV* gene, starting at the *Bam*HI site (nucleotide No. 2747 in SEQ ID No: 3) up to the *Cla*I site at the end of the insert downstream of the *apxIV* gene. This plasmid was designated pROK1. Using

oligonucleotides APXIVAHIS1-L (5'-AGCCATATGGGCGATTTAAATTTCAG-3') (SEQ ID NO: 10) and APXIVAHIS1-R (5'-TATGGATCCTCCGTGCTTCTGAGC-3') (SEQ ID NO: 11) and DNA from plasmid pROK1 as a template, a DNA fragment of 2.1 kb was amplified (see fig. 4) containing the region from bp 3520 to 5643 in *apxIV_var3* (SEQID 3) flanked with *Nde*I and *Bam*HI restriction sites at the 5' and 3' end respectively. After cloning of the *Nde*I/*Bam*HI digested PCR fragment in expression vector pETHIS-1, digested with the same enzymes, a plasmid designated pJFFapxIV6/10his-1 was obtained. Plasmid pETHIS-1 is a derivative of pET14b (Novagen Inc., Madison, Wi.) where the multiple cloning site has been extended and a region encoding a histidine decamer has been inserted. Consequently, The pJFFapxIV6/10his-1 plasmid contains a translational fusion encoding a histidine hexamer, followed by amino acid residues 653 up to 1360 from SEQID 4, followed by a histidine decamer, under the control of a T7 promoter. The plasmid was transferred to *E. coli* strain HMS174(DE3) with pLysS, which contains an IPTG inducible T7 RNA polymerase gene as well as the T7 lysozyme gene for increased stability. The strain was grown in LB medium containing 25 mg/ml of chloramphenicol and 100 mg/ml of ampicillin, up to an OD_{650} of 0.5, and induced with isopropyl-b-D-thiogalactopyranoside at a concentration of 10 mM. After the addition of IPTG, the cells were incubated at 37°C for 2.5 hours, the cells were harvested by centrifugation, and fusion protein with the expected size of 80 kD was isolated in the form of inclusion bodies. The inclusion bodies were solubilized in a solution of 6M guanidine hydrochloride, 300 mM NaCl and 50 mM NaH_2PO_4 at pH 8.0 and the 80 kD fusion protein was further purified by Immobilised Metal Affinity Chromatography (IMAC) (Schmitt et al., Molecular Biology Reports 18;223-230, 1993) using Ni^{2+} chelated columns (Qiagen AG, Basel). Pure protein was eluted from the column at pH 5.0. Pooled fractions were dialysed against a solution of 300 mM NaCl and 50 mM NaH_2PO_4 at pH 8.0. A rabbit was immunised with 500 mg of the polyhistidine fusion product, mixed with 1

volume of Complete Freunds Adjuvant (Difco Labs, Detroit, MI). A booster dose of the same amount, mixed with incomplete Freund Adjuvant was given 3 weeks later. Four weeks after the booster, the rabbit was bled and a hyperimmune serum comprising anti-ApxIV toxin antibodies , designated serum 522-409, was obtained.

Example 4:

Expression of apxIV genes in *in vitro* grown *A. pleuropneumoniae*

The *A. pleuropneumoniae* reference strain from serotype 1 was grown in Columbia broth supplemented with 10 mg/ml of b-NAD and harvested as described (Beck et al., J. Clin. Microbiol., 32;2749-2754, 1994). Adjacent to lanes comprising ApxIA, ApxIIA and ApxIVA-polyhistidine fusion proteins the concentrated culture supernatant was separated by polyacrylamide gel electrophoresis (Laemmli, Nature 227:680-685, 1970) and subjected to a Western blotting procedure (Towbin et al., Proc. Natl. Acad. Sci. USA 76:4350-4354, 1979). The Western blot was reacted with anti-ApxIA- and anti-ApxIIA monoclonal antibodies as described by Beck et al., (J. Clin. Microbiol., 32;2749-2754, 1994), and with anti-ApxIV serum 522-409 (see example 3). The isolated RTX toxin fraction of serotype 1 clearly contains ApxIA and ApxIIA. The presence of ApxIVA could not be demonstrated (see fig. 5).

Example 5:

Expression of apxIV genes in *A. pleuropneumoniae in vivo* during infection

A polyacrylamide gel containing the 80 kD polyhistidine-ApxIV_var3 fusion protein (see example 3) was transferred to a nitrocellulose membrane. The membrane was divided into strips which were reacted with (100-fold dilutions of) convalescent field sera against serotype 1 or sera from a pig, experimentally infected with the serotype 1 reference strain (Frey and Nicolet, Vet.

Microbiol., 28;61-73, 1991). The reaction was visualised using alkaline phosphatase-labelled conjugate against rabbit IgG (Kirkegaard Perry Inc., Gaithersburg, Md.) and NBT (4-Nitrobluetetrazolium chloride) and BCIP (5-Bromo-4-chloro-3-indolyl-phosphate) colour development (see fig. 6). The serotype 1 field sera, as well as serum from the experimentally infected pig react with the 80 kD polyhistidine-ApxIV_var3 protein. This indicates that the ApxIV protein actually is expressed, is antigenic and induces anti-ApxIV toxin antibodies during *A. pleuropneumoniae* infection in pigs.

Example 6:

Presence of *apxIV* genes in all *A. pleuropneumoniae* serotypes and the absence thereof in non-*pleuropneumoniae Actinobacillus*-strains using Southern blotting

To investigate the presence of the *apxIV* gene in the various *A.pleuropneumoniae* serotypes and related bacteria, three probes were made (see fig. 4). Probe APXIVA is described in example 1. Probe APXIVA2 contains the 2015 bp DNA fragment between the *Bam*HI and *Nru*I sites. The 758 bp probe APPIVA1 was made by PCR amplification with oligos APPIV1-L (5'-GGGACAGTGGCTCAATTAAG-3') (SEQ ID NO: 12) and (APPIV1-R (5'-AGCTGTAAACTCCACCAACG-3') (SEQ ID NO: 12). All probes were labelled with Digoxigenin-11-dUTP (Boehringer Mannheim) according to the protocol of the manufacturer and hybridised with Southern blots containing *Cla*I digested chromosomal DNA of all *A. pleuropneumoniae* reference strains and the HV114 field strain, *Actinobacillus suis* (ATCC 15558), *Actinobacillus rossii* (ATCC 27072) and *Actinobacillus equuli* (ATCC 19392). All three probes react similarly (see fig. 7 for the results with the APXIVA2 probe). All *A. pleuropneumoniae* strains react, whereas no hybridisation is observed with the *A. suis*, *A. equuli* and *A. rossii* strains.

Example 7:

Presence of *apxIV* genes in *A. pleuropneumoniae* and related strains using PCR amplification

With 50 ng of chromosomal DNA from the various *A. pleuropneumoniae* serotypes, other *Actinobacillus* species and *P. haemolytica* as templates, and primers APXIVA-1L (5'-TGGCACTGACGGTGATGA-3') (SEQ ID NO: 8) and APXIVA-1R (5'-GGCCATCGACTCAACCAT-3') (SEQ ID NO: 9) PCR amplification was performed. After analysis of the products on an agarose gel, products with the expected size of 442 bp were observed in all *A. pleuropneumoniae* samples, but in none of the other *Actinobacillus* species (fig. 8). This indicates that in addition to the results in example 6, also PCR could be used to discriminate *A. pleuropneumoniae* from other *Actinobacillus* species.

Example 8:

Overexpression of ApxIV-var1 polyhistidine fusion protein.

Starting with plasmid pROK-7 (see example 1) as a template and oligonucleotides APX4/II5-L (5'-CGCCATATGACAAAATTAACTATGCAAG) (SEQ ID NO: 14) and APX4II6-R (5'-CGCGAATTCAGCGACACAAGAGATAT) (SEQ ID NO: 15) as PCR-primers, a PCR fragment was amplified. A sufficient amount of this fragment was then digested with restriction enzymes NdeI and EcoRI and cloned in expression vector pETHIS-1, digested with the same enzymes as described in Example 3. From the resulting plasmid, designated pJFFApxIVA1His1, a 206 kD polyhistidine fusion protein (MW determined in PAGE) of 1841 amino acid residues was overexpressed in E. coli as described in Example 3. The protein is encoded in the coding region spanning nucleic acid no. 1132 to 6546 as depicted in SEQ ID NO: 5. The amino acid sequence of the protein in given in SEQ ID NO: 6. In Western blot this product was shown to

react with specific anti-ApxIV serum 522-409 (antiserum described in example 3).